REMARKS

Claims 1, 6-18 are pending in the current application. Claim 1 is in independent form. Claims 7 and 14-18 have been withdrawn. Claims 1, 8-12 and 14-17 have been amended. In view of the above amendments and following remarks, favorable reconsideration and allowance of the present application is respectfully requested.

Initially, Applicants appreciate the Examiner's acknowledgment that all certified copies pertaining to foreign priority claimed under 35 U.S.C. § 119 have been received, the acceptance of the formal drawings filed on September 28, 2004 and the indication that the references (with the exception of the Cheng QIAN et al.) submitted in the Information Disclosure Statement filed on September 28, 2004 have been considered.

I. INFORMATION DISCLOSURE STATEMENT

With regard to the reference entitled "Induction of Sensitivity to Ganciclovir in Human Hepatocellular Carcinoma Cell by Adenovirus-Mediated Gene Transfer Herpes Simplex Virus Thymidine Kinase" by Cheng QIAN et al., Applicants submit that the year of the reference, 1995, is provided on the bottom left hand side of the first page. A copy of the first page showing the date of 1995 is attached.

As such, Applicants respectfully request consideration of the QIAN reference. Should the Examiner decide to consider the reference, Applicants have enclosed another PTO Form 1449 listing the QIAN reference with the date of 1995.

II. 35 U.S.C. §112, SECOND PARAGRAPH REJECTION

Claims 1-6 and 8-13 stand rejected under 35 U.S.C. §112, first paragraph because the Specification allegedly does not reasonably provide enablement for any and all nanoparticles as well as "a drug" for the "treatment" of any and all hepatic diseases in humans as claimed. Applicants respectfully traverse the rejection.

With regard to the claims, Applicants submit that the claims have been amended to include only arrangements consistent with the Examples provided in the present application.

With regard to the results shown in Fig. 3, Applicants submit that the control experiment was conducted was conducted at the time of the filing of the present application by the Applicants, as shown in the attached Appendix A.

As shown (a) and (b) of Figure 1 of Appendix A, the fact that no drug was administered to the WiDr transplant rat and the NUE transplant rat did not affect tumor growth.

Thus, as shown in Fig. 3 of the present application and supported by Appendix A, the difference in the effect of retraction of the tumor between the WiDr transplant rat and the NUE transplant rat is due to the effectiveness of the drug according to example embodiments.

For at least these reasons, Applicants respectfully request that the Examiner reconsider and withdraw the §112, first paragraph rejection to claims 1-6 and 8-13.

III. CITED ART GROUNDS OF REJECTION

Claims 1-4 and 8 stand rejected under 35 U.S.C. §102(b) as being anticipated by WO 00/46376, the English translation being Hildt et al. (hereinafter "Hildt"), U.S. Patent No. 7,018,826; claims 1-5 and 8 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Hildt and Xiang-Ling et al. (hereinafter "Xiang-Ling"), Killing Effects of Ganciclovir on Human Pulmonary Adenocarcinoma Cell A549 Transduced with HSV1-TK Gene *in vitro* and *in vivo*. Applicants respectfully traverse the rejections.

A. INDEPENDENT CLAIM 1

Amended independent claim 1 is directed to a drug including hollow nanoparticles of a particle-forming protein having a hepatitis B virus surface-antigen protein, the hollow nanoparticles "encapsulating a substance to be transferred to a cell for treatment of hepatic cancer, and the substance including a thymidine kinase (HSV1tk) gene derived from simple herpes virus." Applicants submit that the art relied upon in the rejection fails to teach, or suggest, the above features of amended independent claim 1.

i. THE COMBINATION OF HIDLT AND XIANG-LING

Applicants submit that Hidlt merely discloses that modified HBV vectors bind to hepatic cells. Furthermore, it is unclear how HBV is modified to produce the "modified" HBV vectors.

Thus, Hidlt fails to teach, or suggest, a drug including nanoparticles "encapsulating a substance...the substance including a

thymidine kinase (HSV1tk) gene derived from simple herpes virus" as recited in amended independent claim 1.

Xiang-Ling is directed to the effects of cancer retraction of the HSV-TK gene. Xiang-Ling merely shows the pharmacologic effect of the HSV-TK gene.

Applicants submit that Hidlt and Xiang-Ling, fail to disclose, or suggest, a drug that includes hepatitis B virus surface-antigen protein (HbsAg) nanoparticles encapsulating a HSV-TK gene.

Accordingly, Applicants submit that Hidlt in view of Xiang-Ling fails to teach, or suggest, hollow nanoparticles of a particle-forming protein having a hepatitis B virus surface-antigen protein, the hollow nanoparticles "encapsulating a substance to be transferred to a cell for treatment of hepatic cancer, and the substance including a thymidine kinase (HSV1tk) gene derived from simple herpes virus" as recited in amended independent claim 1.

As such, Applicants respectfully request that the Examiner reconsider, and withdraw the rejection to amended independent claim 1, and claims 6 and 8-13, at least by virtue of their dependency on independent claim 1.

IV. PROVISIONAL DOUBLE PATENTING REJECTION

Claims 1-5 and 8 stand provisionally on the ground of non-statutory obviousness-type double patenting over claims 1 and 2 of copending Application No. 10/509,247, Patent Publication No. 2005/0181064; claims 1-6 and 8-13 stand provisionally rejected on the ground of non-statutory obviousness-type double patenting over claims 1, 3, 4, 6, and 8-12 of copending Application No. 10/509,248, Patent Publication No. 2006/0165726; claims 1-6 and 8-13 stand provisionally rejected on the ground of non-statutory obviousness-type double patenting over claims 1, 8 and 14-16 of copending Application No. 10/509,249, Patent Publication No. 2006/0088536; and claims 1-3 stand provisionally rejected on the ground of non-statutory obviousness-type double patenting over claims 1, 4 and 9 of copending Application No. 10/529,749, Patent Publication No. 2006/00292118.

Applicants note the Examiner's position. However, Applicants submit that until either the present application, or one of U.S. Patent Application Nos. 10/509,247, 10/509,248, 10/509,249, 10/529749, issues into a patent (as acknowledged by the provisional status of the rejection), Applicants submit that the rejections are premature.

V. Withdrawn Claims 7 and 14-18

By the present Amendment, Applicants submit that withdrawn claims 14-17 have been amended to dependent from independent claim 1. Thus, upon the allowance of independent claim 1, Applicants respectfully request that the Examiner rejoin, and allow, all of withdrawn claims 7 and 14-18.

CONCLUSION

Accordingly, in view of the above, reconsideration of the rejections and allowance of each of claims 1 and 6-18 in connection with the present application is earnestly solicited.

Should there be any matters that need to be resolved in the present application; the Examiner is respectfully requested to contact the undersigned at the telephone number below.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 08-0750 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

HARNESS, DICKEY, & PIERCE, P.L.C.

By _____
Donald J. Daley, Reg. No. 34,313

P.O. Box 8910
Reston, Virginia 20195
(703) 668-8000

DJD/CDW:psy

Enclosures:

Appendix A (eight (8) pages)
PTO Form 1449 (one (1) sheet)
Cheng QIAN et al., "Induction of Sensitivity to Ganciclovir in Human Hepatocellular Carcinoma Cell by Adenovirus-Mediated Gene Transfer Herpes Simplex Virus Thymidine Kinase" (one (1) page)

Cancer Gene Therapy (2006) 00, 00–00
 0929-1903/06 $30.00
www.nature.com/cgt

ORIGINAL ARTICLE

Gene therapy of liver tumors with human liver-specific nanoparticles

Y Iwasaki[1], M Ueda[1], T Yamada[2], A Kondo[3], M Seno[4], K Tanizawa[2], S Kuroda[2], M Sakamoto[5] and M Kitajima[1]

[1]*Department of Surgery, School of Medicine, Keio University, Tokyo, Japan;* [2]*Institute of Scientific and Industrial Research, Osaka University, Osaka, Japan;* [3]*Faculty of Engineering, Kobe University, Kobe, Hyogo, Japan;* [4]*Graduate School of Natural Science and Technology, Okayama University, Okayama, Japan and* [5]*Department of Pathology, School of Medicine, Keio University, Tokyo, Japan*

The development of safe and efficient liver-specific gene delivery approaches offers new perspectives for the treatment of liver disease, in particular, liver cancer. We evaluated the therapeutic potential of hepatotropic nanoparticles for gene therapy of liver cancer. These nanoparticles do not contain a viral genome and display the hepatitis B virus L antigen, which is essential to confer hepatic specificity. It has not been shown whether a therapeutic effect could be obtained using L nanoparticles in a human liver cancer xenograft model. Rats bearing human hepatic (NuE) and non-hepatic tumors were injected with L nanoparticles containing a green fluorescent protein (GFP) expression plasmid. GFP expression was observed only in NuE-derived tumors but not in the non-hepatic tumor. The potential for treatment of liver tumors was analyzed using L nanoparticles containing the herpes simplex virus thymidine kinase gene, in conjunction with ganciclovir pro-drug administration. The growth of NuE-derived tumors in L particle-injected rats was significantly suppressed, but not of the non-hepatic tumor control. In summary, this is the first demonstration that nanoparticles could be used for delivery of therapeutic genes with anti-tumor activity into human liver tumors. This intravenous delivery system may be one of the major advantages as compared to many other viral vector systems.
Cancer Gene Therapy (2006) **0,** 000–000. doi:10.1038/sj.cgt.7700990

Keywords: HBsAg L nanoparticle; gene therapy; liver tumor; HSV-*tk*/GCV system

Introduction

Gene therapy is recognized as one of the most promising approaches for treatment of serious diseases, including monogenic diseases, infectious diseases and cancer.[1,2] About 60% of the clinical trial protocols for gene therapy have addressed the treatment of cancer, and a number of preclinical studies are ongoing *in vitro* and *in vivo*. Many types of therapeutic genes have been investigated, and some of them have shown anti-tumor effects. Suicide genes are one such class of therapeutic genes.[3,4] Tumor cells expressing suicide genes become sensitive to pro-drugs, and prodrug treatment can be used to kill the tumor cells specifically. Herpes simplex virus thymidine kinase (HSV-*tk*) gene and ganciclovir (GCV) form one of the more important suicide gene/prodrug combinations. The use of various vectors carrying HSV-*tk* has been reported in treatment of malignant glioma,[4,5] head and neck cancer,[6] colorectal cancer[7] and prostate cancer.[8]

Correspondence: Dr M Ueda, Department of Surgery, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku, Tokyo 160-8582, Japan. E-mail: m_ueda@sc.itc.keio.ac.jp
Received 19 December 2005; revised 18 June 2006; accepted 19 August 2006

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy with a rising incidence worldwide.[9,10] Unresectable HCC lack effective therapy[9,10] and new therapeutic modalities are urgently needed. The transfer of therapeutic genes to liver tumors or to the peritumoral tissue is particularly attractive for the potential treatment of liver cancer and metastasis.[11,12] HCC is also a target of the HSV-*tk*/GCV system.[13–16] However, non-specific expression of suicide genes in non-target cells may cause undesirable adverse effects, such as bone marrow suppression,[17] and for such therapeutic genes to exert their function in a target-specific manner, it is important to specifically deliver them into the target cells or tissues while obviating inadvertent gene transfer into non-target cells.

Various viral vectors, including retroviral, lentiviral, adeno-associated viral (AAV), herpes viral and adenoviral vectors are currently available for gene therapy and are being explored for (liver) cancer gene therapy in particular.[13–16] These vectors do not have target selectivity which reflects the broad tropism of the viruses from which they are derived. Consequently, many vectors wind up transducing non-target cells which reduces the overall efficacy of the gene therapy approach.[18] Moreover, this lack of targeting specificity may cause unexpected adverse

effects, such as bone marrow suppression, due to the inadvertent transgene expression in non-target cells.[17] Although the current vector technologies are being improved further, the development of alternative novel vector systems with improved targeting specificity, higher transduction efficiencies and improved safety is warranted.

We have recently described a novel gene delivery approach based on hepatotropic nanoparticles.[19] These nanoparticles are engineered to display the hepatitis B virus surface L antigen (HBsAg) on their surface and are devoid of viral genomes,[20] which obviates potential safety concerns related to the emergence of replication-competent viruses. These nanoparticles possess high gene transfer efficiency and show high specificity to human liver cells.[19,21] We have succeeded in loading several different genes into the particles, and then transferring these genes into human liver cells *in vitro* and *in vivo*. In addition, L nanoparticles are easily produced by recombinant yeast cells and large-scale production of the particles has already been established,[20,22] whereas large-scale manufacturing of clinical-grade viral vectors remains a significant obstacle that hampers its clinical implementation.[23,24]

It is not known whether nanoparticles could be used successfully for targeted delivery of therapeutic genes with anti-tumor activity into human liver tumors. As a proof-of-concept and as a logical extension of our prior work,[19] we have therefore investigated the distribution of L nanoparticles in a human liver cancer xenograft rat model, using a green fluorescent protein (GFP) expression plasmid as a reporter, and analyzed their potential for treatment of liver tumors using the HSV-*tk*/GCV system.

Materials and methods

Purification and assays for the L protein from the yeast

HBsAg L nanoparticles were prepared from the yeast cells, *Saccharomyces cerevisiae* AH22R⁻, carrying HBsAg L protein expression plasmid pGLDLIIP39-RcT.[20] The whole cell extract of the recombinant yeast was fractionated with PEG 6000, and separated twice by CsCl isopycnic ultracentrifugation and once by sucrose density gradient ultracentrifugation, as described previously.[20,22] The level of the HBsAg L proteins in the yeast cell extracts were determined by silver-stained SDS-PAGE. The S-antigenic activity in the cell extracts was determined by the IMx system (Abbott Laboratories) in conjunction with a microparticle enzyme immunoassay (MEIA) in accordance with the manufacturer's instructions.

Cell cultures

Two cancer cell lines were used for the *in vivo* studies. A spontaneously immortalized cell line derived from a fetal liver cell, NuE,[25] was kindly provided by Professor T Tadakuma (National Defense Medical College, Japan). Hepatocyte specific antigen, keratins and AFP were examined immunohistochemically in this cell line. Hepatocyte specific antigen and keratins were positive, but AFP was negative in NuE (data not shown). We confirmed the growth of tumor when we implanted these cells into mice or rats. We used this transformed hepatocyte cell line as a human liver tumor cell line. The human colon cancer cell line, WiDr, was obtained from American Type Culture Collection (Rockville, MD, USA). NuE cells were maintained in RPMI 1640 medium (Sigma Chemical Co., St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA). WiDr cells were maintained in Dulbecco's modified eagle's medium (DMEM; Sigma) supplemented with 10% FBS. All cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Preparation of L nanoparticles

Mammalian GFP expression plasmid (pTB701-hGFP)[26] (20 μg) under the control of the SV40 promoter or a plasmid expressing HSV-*tk* (pGT65-hINF-alpha) (InvivoGen, San Diego, CA, USA) (20 μg) under the control of the CMV promoter was mixed with purified L nanoparticles (100 μg of protein: 500 μl of 200 μg/ml) in phosphate-buffered saline (PBS; pH 7.2), and then packaging of these genes into the L nanoparticles was performed by electroporation (Gene Pulser II System; Bio-Rad Laboratories, Hercules, CA, USA), as described previously.[19] All experiments were carried out using these gene-containing nanoparticles.

Experimental animals

For the tumor gene therapy study using L nanoparticles, BALB/c nude mice (*nu/nu*, 5 weeks of age, male) and F344/N nude rats (*rnu/rnu*, 5 weeks of age, male) were purchased from CLEA JAPAN. All experimental procedures in this study were conducted according to the guideline of the Animal Care Committee of Keio University.

Morphological analysis

Subcutaneous tumor volume was estimated according to Carlsson's formula,[27] Hence, the largest (a) and smallest (b) superficial diameters of the tumor were measured 0, 5, 7, 9 and 12 days after transplantation of the tumors, and then the volume (V) of the tumor was calculated ($V = a \times b \times b/2$). Statistical analysis was performed using Student's t test. All values were expressed as means $\pm$ s.d.

Histological analysis

The rats that received L nanoparticles were killed 7 days after injection and tumors, brains, hearts, lungs, livers, spleens, kidneys, adrenal glands, intestines and skeletal muscles were isolated. These tissues were fixed in 4% neutralized formaldehyde, according to the method of Kamovsky,[28] and embedded using a Technovit 7100 kit (Heraeus Kulzer GmbH, Wehrheim, Germany). The blocks were sectioned at 5-μm thickness, and then GFP fluorescence in each section was observed with fluorescent microscopy (Nikon ECLIPSE E1000 with a fluorescence system) and with a laser scanning confocal microscope.

L nanoparticles transfer and prodrug treatment of rats
GCV (InvivoGen, San Diego, CA, USA) as a prodrug for HSV-*tk* was dissolved in sterile water, according to the manufacturer's instruction. Continuous GCV administration (50 mg/kg/day) was performed using an osmotic pump (DURECT Co., Cupertino, CA, USA) on the back of the tumor-bearing rats ($n = 5$), starting from the first day after transplantation. L nanoparticles for the expression of HSV-*tk* were injected on day 5 after transplantation. The effect of GCV/HSV-*tk* was estimated by measurement of tumor volume.

Results

Expression and characterization of L nanoparticles
L nanoparticles were overexpressed in *S. cerevisiae* AH22R$^-$ carrying L protein expression plasmid pGLDLIIP39-RcT.[20] After fractionation of the lyses by CsCl and sucrose gradient ultracentrifugation, L nanoparticles in the various fractions were separated by SDS-PAGE which was stained with silver reagent, as described previously.[20,22] The S-antigenic activity in the cell extracts was determined by the IMx system (Abbott Laboratories) in conjunction with a microparticle enzyme immunoassay (MEIA) in accordance with the manufacturer's instructions.

Distribution of L nanoparticles in the rat model
In pre-clinical experiments, it is important to assess the biodistribution and safety of gene transfer vectors *in vivo*. We adopted a xenograft rat model bearing hepatic (NuE) and non-hepatic (WiDr) cell-derived tumors. About 1×10^7 tumor cells (both NuE and WiDr) were subcutaneously injected into the rear flank of the mice. To normalize the size of the tumors, tumor-bearing mice were killed, and then both NuE- and WiDr-derived tumors (5 mm in diameter) were subcutaneously transplanted into the rear flank of the same nude rat. Rats bearing large enough tumors (NuE tumor: $434.1 \pm 134.8\,cm^3$, WiDr tumor: $400.2 \pm 146.0\,cm^3$) were injected with 100 μg (500 μl) of L nanoparticles carrying 20 μg of expression plasmids, via the tail vein ($n = 5$). As a negative control, rats were injected with PBS. The rats were killed 7 days after L nanoparticle or PBS injection. The tumors (NuE and WiDr-derived), livers, spleens and kidneys were extracted, and the morphological differences and the weights of these tissues were compared between the L nanoparticle and PBS control groups. There were no obvious differences in growth rates or tumor sizes of the tumors of rats injected with L nanoparticles containing GFP expression plasmids versus PBS controls (Figure 1a and b, Table 1) indicating that the *in vivo* transfection *per se* does not affect tumor growth. In addition, histological examination revealed no apparent histologic abnormalities in liver, lung, brain, kidney, heart, adrenal gland, skeletal muscle, spleen and intestine of tumor-bearing rats injected with L nanoparticles *versus* PBS controls (Figure 2, Table 1). Moreover, intravenous delivery of L nanoparticles in the tumor-bearing animals did not significantly alter body weight or the weight of the individual organs (Table 1), nor was there any detectable change in blood platelet count and aminotransferase level in blood as markers of hepatic acute toxicity (data not shown). Collectively, these data indicate that there was no detectable toxicity of the L nanoparticles in this rat model based on histologic and macroscopic analysis, liver transaminase levels and hematologic parameters, which further corroborates their relative safety.

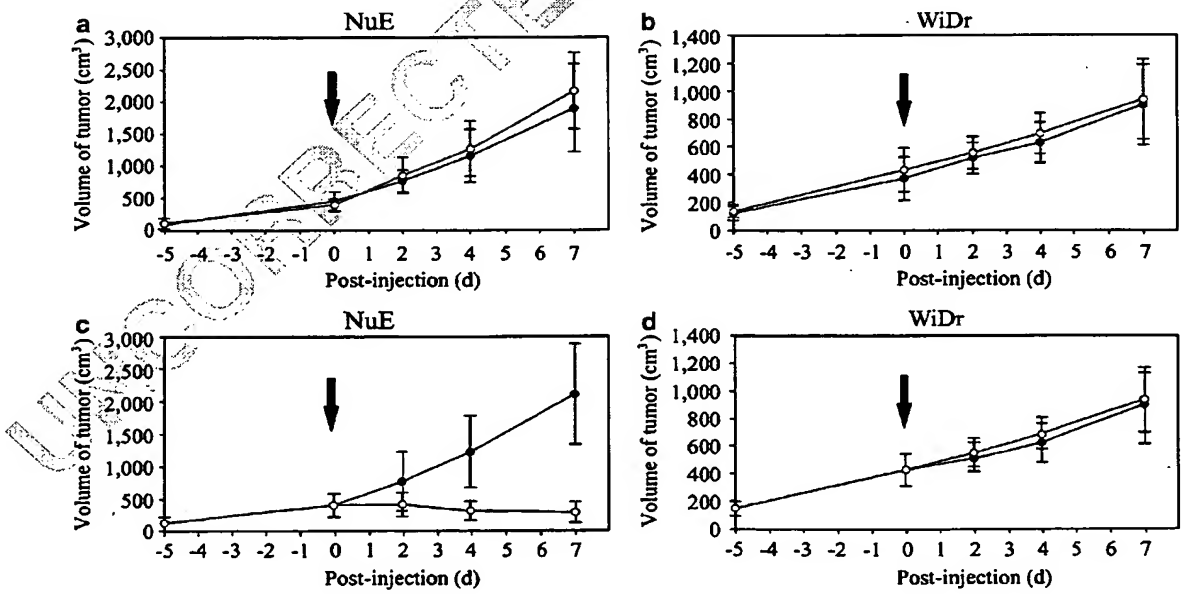

Figure 1

GFP fluorescence was measured to investigate the distribution of L nanoparticles, after killing the rats 7 days post-injection. Following injection of the GFP nanoparticles, fluorescence was observed in the hepatic NuE-derived tumors only but not in the non-hepatic WiDr-derived tumors (Figures 3, 4a and b). This underscores the hepatic specificity of the nanoparticles. No significant fluorescence was detectable in the NuE- or WiDr-derived tumors from rats injected with PBS control (Figure 4c and d). Furthermore, there was no detectable fluorescence in rat liver (Figures 2 and 3), indicating that the nanoparticles were specific for human liver tumor cells, whereas rat hepatocytes were refractory to L nanoparticle-mediated gene transfer. In addition, there was no evidence of transfection into non-hepatic tissues since only aspecific background fluorescence was apparent in brain, lung, kidney, heart, spleen, adrenal gland, intestine and skeletal muscle (Figure 2). This fluorescence, which could be observed through both GFP and Cy3 filters, was inherent to the examined tissue and undistinguishable from PBS controls.

Table 1

	L (+)	*L (−)*	P
Body weight (g)	179.3±9.6	180.3±15.8	NS
Tumor (NuE) (g)	12.2±1.7	11.8±1.8	NS
Tumor (WiDr) (g)	7.0±1.0	7.1±1.0	NS
Liver (g)	10.7±0.7	10.3±0.5	NS
Spleen (g)	1.0±0.1	1.0±0.1	NS
Kidney (lt) (g)	1.1±0.1	1.1±0.1	NS

Abbreviation: NS, nonsignificant.

Suicide gene therapy with L nanoparticles

The biodistribution studies based on GFP reporter gene expression indicate that the L nanoparticles are well suited to achieve targeted gene delivery and cell-specific expression in liver tumor. To determine whether L nanoparticles could be used for liver tumor-specific gene

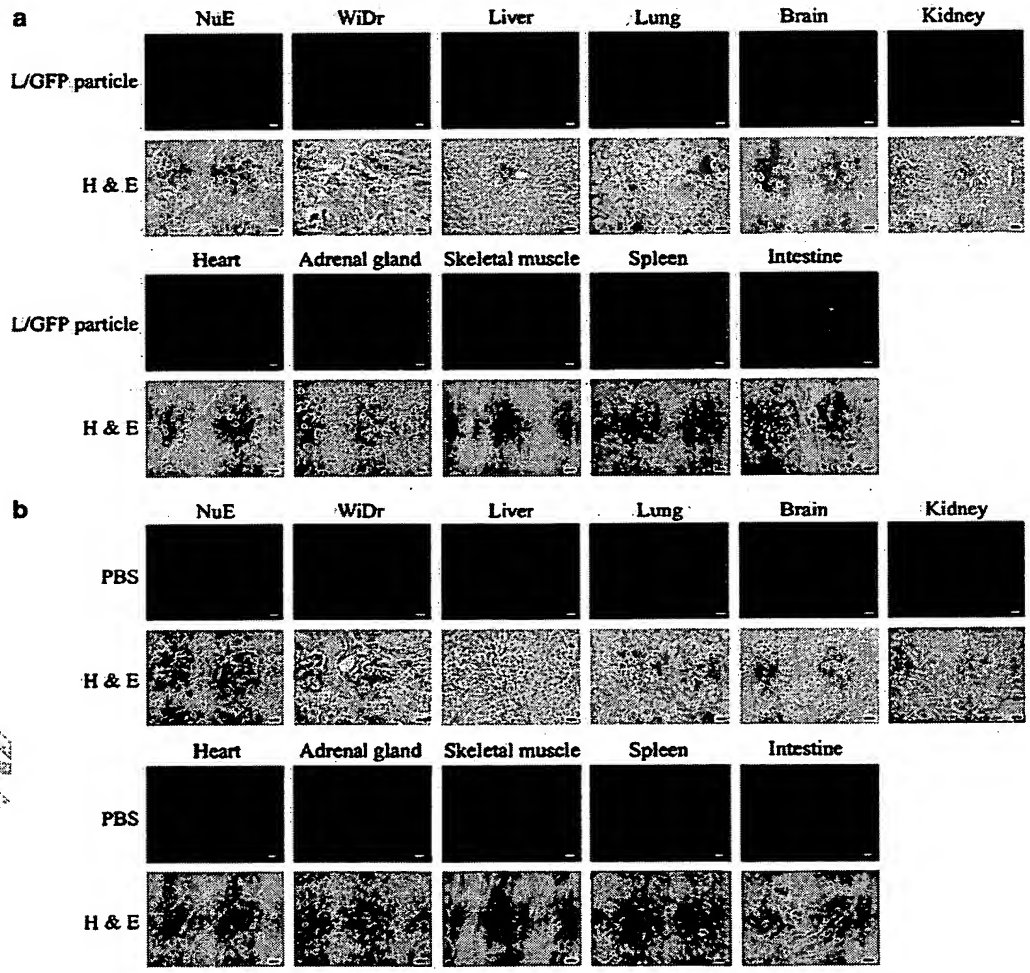

Figure 2

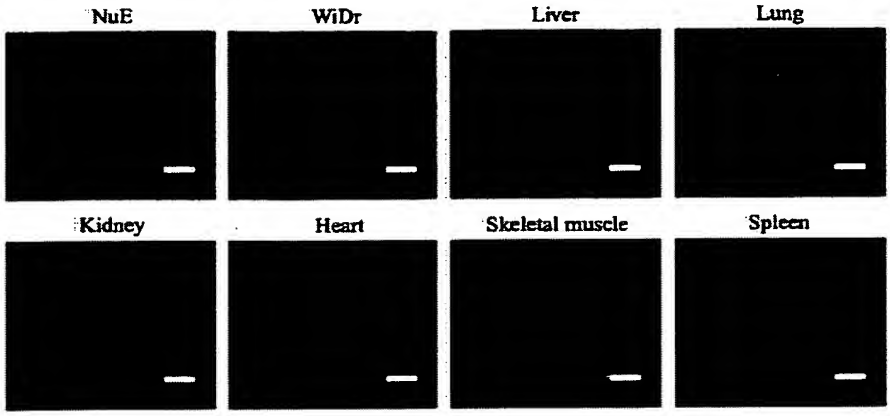

Figure 3

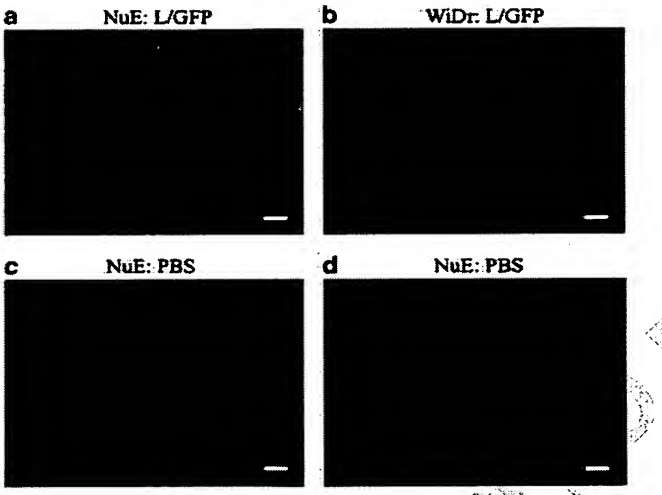

Figure 4

therapy, it was important to demonstrate that a therapeutic effect could be obtained by targeted delivery of therapeutic genes into the liver tumors. The therapeutic potential of the nanoparticles was determined by loading the particles with the herpes simplex virus thymidine kinase (HSV-*tk*) gene and by assessing the anti-tumor effect following ganciclovir (GCV) pro-drug administration in the rat xenograft tumor model.

Continuous GCV administration (50 mg/kg/day) was performed in rats ($n=5$) bearing NuE hepatic tumors, starting from the first day after transplantation. L nanoparticles containing 20 μg HSV-*tk* expression plasmids were injected intravenously into tumor-bearing rats on day 5 after transplantation. As a control, injection of 20 μg HSV-*tk* plasmids alone was used. The growth of NuE-derived hepatic tumors in L nanoparticle-injected rats was significantly ($P<0.05$) suppressed during the course of the experiment ($456.2\pm124.5\,cm^3$), whereas NuE-derived tumor growth progressed in the control group that only received the HSV-*tk* genes without L nanoparticles ($2120.0\pm778.5\,cm^3$) (Figure 1c). In contrast, there was no tumor regression following nanoparticle/HSV-*tk* and GCV administration in WiDr-derived non-hepatic tumor-bearing rats, compared to the WiDr tumor control group that received only HSV-*tk*/GCV (Figure 1d). Moreover, there was no anti-tumor effect in the NuE and WiDr controls injected with L nanoparticles/GFP (Figure 1a and b). There were no apparent side-effects associated with the nanoparticle transfection, nor was there a significant difference in body or organ weight between the experimental and control groups (data not shown). Hence, these results demonstrate for the first time that L nanoparticles could be used successfully and safely for delivery of therapeutic genes with anti-tumor activity into human liver tumors with unprecedented targeting specificity, resulting in liver tumor-selective expression of the therapeutic gene.

Discussion

In the present study, we investigated the effects of L nanoparticles *in vivo* in a rat xenograft tumor model. The biodistribution of L nanoparticles was determined by analyzing GFP expression in hepatic and non-hepatic tumors and in various tissues. The fluorescence and histologic analysis indicate that GFP expression is restricted to and specific for the transplanted liver tumors (NuE) (Figures 2–4), indicating that nanoparticles are well suited for targeted gene delivery and expression for human liver tumor. In the current study, a detailed histologic analysis was undertaken and the results are consistent with and extend our previous observations regarding L nanoparticle-mediated delivery of GFP or fluorescent compounds in a mouse model.[19] This tropism of the L nanoparticles for human liver tumor reflects the natural tropism of the human hepatitis B virus (HBV) which is primarily determined by the pre-S1 targeting

peptide located at the N-terminal end of the L antigen.[29] The lack of GFP expression in the rat liver following L nanoparticle transfection is consistent with the resistance of rodent hepatocytes to HBV infection.[30] The hepatotropic specificity of the HBV virus and consequently also the L nanoparticles, is determined by the interaction of the HBsAg with an elusive cellular receptor, although several candidate receptors or co-receptors have been proposed, including annexin V[31] and squamous cell carcinoma antigen-1 homolog (SCCA-1).[32] L nanoparticles are well tolerated and do not appear to induce serious adverse effects (Table 1, Figure 2). Furthermore, L nanoparticles by themselves have no effect on tumor suppression or progression (Figure 1).

The lack of viral genomes in the L nanoparticles obviates concerns regarding the possible generation of replication-competent viruses that may arise by homologous recombination, as in the case of early-generation adenoviral vectors and retroviral vectors.[33] Moreover, the safety profile of the L nanoparticles compares favorably compared to the safety of several viral vectors.

The immunogenicity of the L nanoparticles is controversial. The ability for repetitive administration is of paramount importance in the clinical context. Most of the world's population has antibodies against hepatitis B because of prior infection or due to immunization. Hence, the application of L particles would be limited to the patients who have no antibodies against the virus. However, HBsAg immune escape variants exist in chronic hepatitis B patients and eliminating/modifying the immunogenic epitopes from L particles could minimize these limitations.

The HSV-*tk*/GCV paradigm is an attractive anti-cancer gene therapy approach, because it generally provides for a potent anti-tumor response, by virtue of the bystander effect.[5,34] However, the expression of HSV-*tk* in non-target cells may result in unexpected adverse effects, because all cells transduced with HSV-*tk* genes become sensitive to GCV. In studies of anti-HCC therapy, an α-fetoprotein (AFP) promoter or enhancer has been employed to drive HSV-*tk* specifically into HCC cells.[35,36] Although use of an AFP promoter or enhancer induces HCC-selective expression, the transgenes rarely work in non- or low-AFP-producing HCC cells. In this study, we attempted to express HSV-*tk* genes in NuE cells, which are low AFP-producing liver tumor cells.[37] L nanoparticles carrying HSV-*tk* genes were injected into tumor-bearing rats intravenously, and growth of NuE-derived hepatic tumors in the experimental group was suppressed in conjunction with GCV administration (Figure 1c), whereas WiDr-derived non-hepatic tumors showed progression during the course of the experiment (Figure 1d). The suppression of tumor-growth was strictly depending on the L nanoparticles since injection of HSV-*tk*/GCV without nanoparticles had no effect on tumor growth (Figure 1c). These results are consistent with the GFP biodistribution profile (Figures 2 and 3) and indicate that L nanoparticles can specifically deliver therapeutic transgenes into human liver cells. Nanoparticle-mediated targeted gene delivery can enable liver tumor-selective expression while obviating the use of an AFP promoter or enhancer, which may not be ideally suited to target expression in HCC, especially in those cells in which AFP expression is either absent or very low. Nevertheless, it is possible that selective transgene expression in human hepatic tumor cells following nanoparticle-mediated gene transfer could be improved further when promoters are chosen that are expressed at higher levels in cancerous versus normal hepatic tissue, as identified by transcriptional profiling.[38,39] Although it is known that the L nanoparticles also transfect normal human hepatocytes but not murine hepatocytes,[19] it has been shown that only dividing cells are susceptible to phosphorylated GCV.[40] Thus, expression of HSV-*tk* with L particles in quiescent normal hepatocytes followed by GCV treatment might not cause damage to normal hepatocytes but would be restricted to dividing liver tumor cells instead. This would be consistent with recent observations showing strong antitumoral efficacy in the absence of normal liver toxicity following direct intratumoral injection of a lentiviral vector containing the HSV-*tk* gene and GCV treatment.[16] However, this study and our current observations are in contrast with the severe liver dysfunction and mortality that arose following systemically administered hepatotropic adenoviral vectors expressing HSV-*tk* and GCV treatment.[41] These data further confirm that the L nanoparticles may have an improved biosafety profile compared to adenoviral vectors for hepatic gene delivery. Interestingly, intravenous injection of L nanoparticles appeared to show similar efficacy to the intratumoral injection of adenoviral vectors into large tumor nodules.[42]

The current study underscores the potential of hepatotropic nanoparticles for gene therapy of liver tumor, which may pave the way towards a more efficacious and safer hepatic gene delivery approach. Future studies will require safety and efficacy analysis of the L nanoparticles in mouse models with 'humanized' liver[43] in conjunction with potent liver-tumor specific promoters to further refine the selectivity of expression.

Acknowledgements

We thank Takuji Tadakuma (the Department of Parasitology, National Defense Medical College) for the gift of the NuE cell line, and the HBV nanobioparticle team for technical support. This study was supported by the Grants-in-Aid from Ministry of Education, Culture, Sports, Science and Technology, Japan (the 21st century Center of Excellence program and Scientific Research). Thierry VandenDriessche and Marinee KL Chuah are supported by an FWO grant (G.0341.05) and a grant from the Flemish Government (VIB Dotation).

References

1 Friedmann T. Human gene therapy – an immature genie, but certainly out of the bottle. *Nat Med* 1996; 2: 144–147.

2 Verma IM, Somia N. Gene therapy – promises, problems and prospects. *Nature* 1997; **389**: 239–242.
3 Connors TA. The choice of prodrugs for gene directed enzyme prodrug therapy of cancer. *Gene Therapy* 1995; **2**: 702–709.
4 Sandmair AM, Loimas S, Puranen P, Immonen A, Kossila M, Puranen M *et al.* Thymidine kinase gene therapy for human malignant glioma, using replication-deficient retroviruses or adenoviruses. *Hum Gene Ther* 2000; **11**: 2197–2205.
5 Ram Z, Culver KW, Oshiro EM, Viola JJ, DeVroom HL, Otto E *et al.* Therapy of malignant brain tumors by intratumoral implantation of retroviral vector-producing cells. *Nat Med* 1997; **3**: 1354–1361.
6 Morris JC, Wildner O. Therapy of head and neck squamous cell carcinoma with an oncolytic adenovirus expressing HSV-*tk*. *Mol Ther* 2000; **1**: 56–62.
7 Okabe S, Arai T, Yamashita H, Sugihara K. Adenovirus-mediated prodrug-enzyme therapy for CEA-producing colorectal cancer cells. *J Cancer Res Clin Oncol* 2003; **129**: 367–373.
8 Loimas S, Toppinen MR, Visakorpi T, Janne J, Wahlfors J. Human prostate carcinoma cells as targets for herpes simplex virus thymidine kinase-mediated suicide gene therapy. *Cancer Gene Ther* 2001; **8**: 137–144.
9 Okuda K. Hepatocellular carcinoma. *J Hepatol* 2000; **32**: 225–237.
10 Lau WY. Primary liver tumors. *Semin Surg Oncol* 2000; **19**: 135–144.
11 Ruiz J, Qian C, Drozdzik M, Prieto J. Gene therapy of viral hepatitis and hepatocellular carcinoma. *J Viral Hepat* 1999; **6**: 17–34.
12 Qian C, Drozdzik M, Caselmann WH, Prieto J. The potential of gene therapy in the treatment of hepatocellular carcinoma. *J Hepatol* 2000; **32**: 344–351.
13 Gao Ding C, An W, Dai J. Retrovirus-mediated herpes simplex virus thymidine kinase gene therapy approach for hepatocellular carcinoma. *Cell Res* 1999; **9**: 225–235.
14 Su H, Lu R, Ding R, Kan YW. Adeno-associated viral-mediated gene transfer to hepatoma: thymidine kinase/interleukin 2 is more effective in tumor killing in non-ganciclovir (GCV)-treated than in GCV-treated animals. *Mol Ther* 2000; **1**: 509–515.
15 Tsuchiyama T, Kaneko S, Nakamoto Y, Sakai Y, Honda M, Mukaida N *et al.* Enhanced antitumor effects of a bicistronic adenovirus vector expressing both herpes simplex virus thymidine kinase and monocyte chemoattractant protein-1 against hepatocellular carcinoma. *Cancer Gene Ther* 2003; **10**: 260–269.
16 Gerolami R, Uch R, Faivre J, Garcia S, Hardwigsen J, Cardoso J *et al.* Herpes simplex virus thymidine kinase-mediated suicide gene therapy for hepatocellular carcinoma using HIV-1-derived lentiviral vectors. *J Hepatol* 2004; **40**: 291–297.
17 Cao G, Kuriyama S, Gao J, Mitoro A, Cui L, Nagao S *et al.* *In vivo* gene transfer of a suicide gene under the transcriptional control of the carcinoembryonic antigen promoter results in bone marrow transduction but can avoid bone marrow suppression. *Int J Oncol* 1999; **15**: 107–112.
18 VandenDriessche T, Thorrez L, Naldini L, Follenzi A, Moons L, Berneman Z *et al.* Lentiviral vectors containing the human immunodeficiency virus type-1 central polypurine tract can efficiently transduce nondividing hepatocytes and antigen-presenting cells *in vivo*. *Blood* 2002; **100**: 813–822.
19 Yamada T, Iwasaki Y, Tada H, Iwabuki H, Chuah MK, VandenDriessche T *et al.* Nanoparticles for the delivery of genes and drugs to human hepatocytes. *Nat Biotechnol* 2003; **21**: 885–890.
20 Kuroda S, Otaka S, Miyazaki T, Nakao M, Fujisawa Y. Hepatitis B virus envelope L protein particles. Synthesis and assembly in Saccharomyces cerevisiae, purification and characterization. *J Biol Chem* 1992; **267**: 1953–1961.
21 Yu D, Amano C, Fukuda T, Yamada T, Kuroda S, Tanizawa K *et al.* The specific delivery of proteins to human liver cells by engineered bio-nanocapsules. *FEBS J* 2005; **272**: 3651–3660.
22 Yamada T, Iwabuki H, Kanno T, Tanaka H, Kawai T, Fukuda H *et al.* Physicochemical and immunological characterization of hepatitis B virus envelope particles exclusively consisting of the entire L (pre-S1 + pre-S2 + S) protein. *Vaccine* 2001; **19**: 3154–3163.
23 Merten OW, Cruz PE, Rochette C, Geny-Fiamma C, Bouquet C, Goncalves D *et al.* Comparison of different bioreactor systems for the production of high titer retroviral vectors. *Biotechnol Prog* 2001; **17**: 326–335.
24 Snyder RO, Flotte TR. Production of clinical-grade recombinant adeno-associated virus vectors. *Curr Opin Biotechnol* 2002; **13**: 418–423.
25 Hosokawa S, Muramatsu M, Nagaike K. Detection of membrane-bound alpha-fetoprotein in human hepatoma cell lines by monoclonal antibody 19F12. *Cancer Res* 1989; **49**: 361–366.
26 Inouye S, Ogawa H, Yasuda K, Umesono K, Tsuji FI. A bacterial cloning vector using a mutated Aequorea green fluorescent protein as an indicator. *Gene* 1997; **189**: 159–162.
27 Carlsson G, Gullberg B, Hafstrom L. Estimation of liver tumor volume using different formulas - an experimental study in rats. *J Cancer Res Clin Oncol* 1983; **105**: 20–23.
28 Kamovsky MJ. A formaldehyde-glutaraldehyde fixation of high osmolality for use in electron microscopy. *J Cell Biol* 1965; **2**: 137A–138A.
29 Neurath AR, Kent SB, Strick N, Parker K. Identification and chemical synthesis of a host cell receptor binding site on hepatitis B virus. *Cell* 1986; **46**: 429–436.
30 Barker LF, Maynard JE, Purcell RH, Hoofnagle JH, Berquist KR, London WT. Viral hepatitis, type B, in experimental animals. *Am J Med Sci* 1975; **270**: 189–195.
31 Gong ZJ, De Meyer S, van Pelt J, Hertogs K, Depla E, Soumillion A *et al.* Transfection of a rat hepatoma cell line with a construct expressing human liver annexin V confers susceptibility to hepatitis B virus infection. *Hepatology* 1999; **29**: 576–584.
32 Moore PL, Ong S, Harrison TJ. Squamous cell carcinoma antigen 1-mediated binding of hepatitis B virus to hepatocytes does not involve the hepatic serpin clearance system. *J Biol Chem* 2003; **278**: 46709–46717.
33 VandenDriessche T, Collen D, Chuah MK. Biosafety of onco-retroviral vectors. *Curr Gene Ther* 2003; **3**: 501–515.
34 Caruso M, Panis Y, Gagandeep S, Houssin D, Salzmann JL, Klatzmann D. Regression of established macroscopic liver metastases after *in situ* transduction of a suicide gene. *Proc Natl Acad Sci USA* 1993; **90**: 7024–7028.
35 Kaneko S, Hallenbeck P, Kotani T, Nakabayashi H, McGarrity G, Tamaoki T *et al.* Adenovirus-mediated gene therapy of hepatocellular carcinoma using cancer-specific gene expression. *Cancer Res* 1995; **55**: 5283–5287.
36 Su H, Lu R, Chang JC, Kan YW. Tissue-specific expression of herpes simplex virus thymidine kinase gene delivered by adeno-associated virus inhibits the growth of human

hepatocellular carcinoma in athymic mice. *Proc Natl Acad Sci USA* 1997; **94**: 13891–13896.
37 Murayama Y, Tadakuma T, Kunitomi M, Kumai K, Tsutsui K, Yasuda T *et al.* Cell-specific expression of the diphtheria toxin A-chain coding sequence under the control of the upstream region of the human alpha-fetoprotein gene. *J Surg Oncol* 1999; **70**: 145–149.
38 Neo SY, Leow CK, Vega VB, Long PM, Islam AF, Lai PB *et al.* Identification of discriminators of hepatoma by gene expression profiling using a minimal dataset approach. *Hepatology* 2004; **39**: 944–953.
39 Breuhahn K, Vreden S, Haddad R, Beckebaum S, Stippel D, Flemming P *et al.* Molecular profiling of human hepatocellular carcinoma defines mutually exclusive interferon regulation and insulin-like growth factor II overexpression. *Cancer Res* 2004; **64**: 6058–6064.
40 Chen SH, Shine HD, Goodman JC, Grossman RG, Woo SL. Gene therapy for brain tumors: regression of experimental gliomas by adenovirus-mediated gene transfer *in vivo*. *Proc Natl Acad Sci USA* 1994; **91**: 3054–3057.
41 van der Eb MM, Cramer SJ, Vergouwe Y, Schagen FH, van Krieken JH, van der Eb AJ *et al.* Severe hepatic dysfunction after adenovirus-mediated transfer of the herpes simplex virus thymidine kinase gene and ganciclovir administration. *Gene Therapy* 1998; **5**: 451–458.
42 Gerolami R, Cardoso J, Lewin M, Bralet MP, Sa Cunha A, Clement O *et al.* Evaluation of HSV-*tk* gene therapy in a rat model of chemically induced hepatocellular carcinoma by intratumoral and intrahepatic artery routes. *Cancer Res* 2000; **60**: 993–1001.
43 Tateno C, Yoshizane Y, Saito N, Kataoka M, Utoh R, Yamasaki C *et al.* Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am J Pathol* 2004; **165**: 901–912.

NPG.CGT.7700990